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The architecture of the IgG anti-carbohydrate repertoire in primary antibody deficiencies (PADs)

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Abstract:

Immune system failure in primary antibody deficiencies (PADs) has been linked to recurrent infections, autoimmunity and cancer, yet clinical judgment is often based on the reactivity to a restricted panel of antigens. Previously, we demonstrated that the human repertoire of carbohydrate-specific IgG exhibits modular organization related to glycan epitope structure. The current study compares the glycan-specific IgG repertoires among different PAD entities. Distinct repertoire profiles with extensive qualitative glycan-recognition defects were observed, characterized by the common loss of Gal α - and GalNAc-reactivity and disease-specific recognition of microbial, self-antigens and tumor-associated carbohydrate antigens. Antibody repertoire analysis may provide a useful tool to elucidate the dimension and clinical implications of the immune system failure in individual patients.

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The architecture of the IgG anti-carbohydrate repertoire in primary antibody deficiencies (PADs)

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KEY POINTS: Repertoire analysis by microarray technology constitutes a powerful tool to evaluate immune system failure in PADs.

ABSTRACT

Immune system failure in primary antibody deficiencies (PADs) has been linked to recurrent infections, autoimmunity and cancer, yet clinical judgment is often based on the reactivity to a restricted panel of antigens. Previously, we demonstrated that the human repertoire of carbohydrate-specific IgG exhibits modular organization related to glycan epitope structure. The current study compares the glycan-specific IgG repertoires among different PAD entities. Distinct repertoire profiles with extensive qualitative glycan-recognition defects were observed, characterized by the common loss of Gal α - and GalNAc-reactivity and disease-specific recognition of microbial, self-antigens and tumor-associated carbohydrate antigens. Antibody repertoire analysis may provide a useful tool to elucidate the dimension and clinical implications of the immune system failure in individual patients.

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INTRODUCTION

Inadequate humoral responses to carbohydrate-structures represent a common feature of primary antibody deficiencies (PADs), despite the pathogenetic heterogeneity of these disorders, involving monogenetic, polygenetic and still unexplained defects^{1,2}. These disorders are associated with a plethora of clinical sequelae including severe and recurrent infections, microbial dysbiosis, autoimmunity, allergy, granulomatous disease and malignancy^{1,3,4}.

The surface of all living cells is glycosylated, whereby the composition varies between cell type⁵, individuals, or species⁶. The prominent exposure of carbohydrate-structures (glycans) on the surface of cells or bacterial capsules, renders them accessible to antibodies, which facilitates the detection and elimination of pathogens or aberrant cells, but also leads to adverse reaction in transfusion/transplantation procedures (blood groups antigens), or to the acute rejection of xenografts (e.g. Gal- α structures). Due to altered expression of biosynthetic enzymes, such as glycosyltransferases or glycosidases, the glycome of cells is often changed under pathological conditions, including cancer⁷. While tumor-associated carbohydrates (TACAs) are exploited as diagnostic markers⁸, there is also evidence for naturally-occurring antibodies to TACAs in healthy individuals⁹.

Insufficient responses to glycan-based vaccines or low titers of isohemagglutinins, antibodies to polysaccharide blood group antigens, are characteristic and diagnostic features of common variable immunodeficiency (CVID), the most frequent symptomatic antibody deficiency diagnosed in adulthood^{10,11}. Patients with specific antibody deficiency (SPAD) exhibit poor responses to structural or capsular polysaccharides of bacteria (e.g. *S. pneumoniae*, *H. influenzae*), despite the presence of normal serum concentrations of IgG, IgM and IgA¹². Antibodies from

89 different IgG subclasses are known to contain different specificities for glycan-
90 structures^{9,13}, which may explain the predisposition of certain patients with IgG
91 subclass deficiency (IgGSD) to infections with encapsulated bacteria¹². However,
92 current knowledge on the failure to raise adequate levels of glycan-specific
93 antibodies in PAD is mainly based on experience with specific bacterial antigens.

94 The clinical assessment of PADs by diagnostic vaccination (e.g.
95 pneumococcal vaccines, *S. typhi* Vi vaccine¹⁴) or the measurement of pre-existing
96 antibody titers (e.g. isohemagglutinins) relies on a restricted number of glycan
97 epitopes, thus providing only a narrow perspective of the actual dimensions of the
98 immunodeficiency. Further disadvantages of diagnostic vaccination include
99 diagnostic delay, laboratory-to-laboratory variation, serotype-specific responses, age
100 differences in antibody responses, or the challenging interpretation of
101 pre-immunization versus post-immunization specific antibody levels in patients not
102 receiving IgG replacement therapy^{10,11,15-18}. The broader assessment of glycan-
103 specific antibodies in patients may better reflect the immune defect and also facilitate
104 treatment decisions, such as regarding life-long IgG replacement therapy.

105 Glycan array technology allows the high-throughput analysis of specific
106 antibody responses to carbohydrate antigens¹⁹⁻²¹. In a previous study using glycan
107 array version 5.1 of The Consortium for Functional Glycomics (CFG) to decipher the
108 IgG repertoire of healthy individuals we found that classes of glycans were
109 recognized with different intensity, depending on the terminal carbohydrate-moiety⁹.
110 Here, we employed glycan array technology to investigate the IgG antibody repertoire
111 of PAD patients in terms of clinically relevant carbohydrate epitopes, including
112 microbial glycans, self- or xenoantigens, and TACAs.

MATERIALS AND METHODS

Study design

This non-randomized study was designed to investigate the human IgG anti-carbohydrate repertoire, in healthy and disease conditions, using glycan array technology combined with a computational system level approach. To this end, sera or purified IgG from healthy individuals or patients, as well as control antibodies were screened on microarrays by the Consortium for Functional Glycomics (CFG) or the US National Center for Functional Glycomics (NCFG).

Patient samples

Human blood was collected from healthy individuals (HD) or patients upon informed and written consent in accordance with the Declaration of Helsinki. All experimental protocols were approved by the local institutional and/or licensing committees (KEK-BE: 148/10 and KEK No. 224/01). Therapeutic naïve patients followed at the University Hospital of Bern from January 2005 to December 2011 were retrospectively identified. Additional sera of therapeutic naïve patients without IgG replacement therapy were provided from B.G. CVID was defined in accordance with the criteria of the Pan-American Group for Immunodeficiency and the European Society for Immunodeficiency²². Inclusion criteria for IgGSD were a normal total IgG concentration with a significant decrease (more than two standard deviations below the mean for the age) in the serum concentrations of one or more IgG subclasses²³ with recurrent episodes of infection. Symptomatic hypogammaglobulinemia (HGG) was defined with decreased total IgG concentration, but not fulfilling criteria for CVID with respect to a reduction of two immunoglobulin isotypes and/or reduced response to vaccination. For diagnostic vaccination the pneumococcal polysaccharide vaccine

(PPV) Pneumovax-23 (MSD, Lucerne, Switzerland) was used in patients with IgG levels at or higher than 4 g/L. Levels of pneumococcal capsular polysaccharide (PCP) IgG > 50 mg/L and for PCP IgG2 > 40 mg/L were considered as normal. A sufficient PPV vaccination response was defined as PCP titers above these levels and/or PCP IgG and IgG2 levels above 100 mg/L and/or a 4-fold increase of post-vaccination PCP IgG and PCP IgG2 titers as detected 4 to 6 weeks after vaccination. SPAD was diagnosed in patients with normal total immunoglobulin and IgG subclass concentrations but impaired pneumococcal polysaccharide vaccine (PPV) response. The characteristics of the different groups are summarized in Table S1. Patients were not stratified for specific infections. The sera from these patients were individually screened in the NCFGv2 glycan array or pooled in the indicated groups and screened in the CFG glycan array. IgG purification was performed by affinity chromatography in Ab SpinTrap columns (GE Healthcare). Pooled sera from patients and healthy donors were directly applied to the columns and the purification procedures performed according to manufacturer instructions. The control IgG preparation (IgG control mix) was prepared by mixing two monoclonal human myeloma proteins, IgG1 λ (67%) and IgG2 κ (33%), purchased from Sigma-Aldrich. This process resulted in a κ/λ ratio of 0.5, which is well within the range found in normal serum (0.26 to 1.65). The concentration of IgG in the different samples was determined using a Behring Turbitimer instrument (Dade Behring). The quality of the isolated antibodies was checked by SDS-PAGE under reducing and non-reducing conditions (Figure S1).

Glycan array analysis

The glycan microarrays from the CFG (<http://www.functionalglycomics.org/static/consortium/resources/resourcecoreh8.shtm>

l) were prepared from amine functionalized glycan structures covalently coupled in microarrays to N-hydroxysuccinimide-derivatized microscope slides as previously described²⁴. On NCFG array version 2 (NCFGv2) carbohydrates were conjugated with the fluorescent linker AEAB, by reductive amination with sodium cyanoborohydrate to form glycan-AEABs (GAEABs). For both arrays, the human sera or purified IgG or IgG control mix, were identically processed and screened at 180µg/ml using a secondary biotinylated anti-human IgG mAb (clone HP-6043) at 5 µg/ml followed by Alexa633-coupled streptavidin⁹. A positive streptavidin control was used. Sample preparation and analysis was performed as indicated on the CFG website (www.functionalglycomics.org). To determine the specific binding to selected glycans, the antibody binding ratio (ABR) was calculated. The computed ABR represents the quotient of the respective sample relative fluorescent units (RFU) and the corresponding IgG control mix RFU. Data are expressed as the mean of RFU or ABR values from six repeated experiments, if not indicated otherwise.

Database search

The identity or characteristics of glycans was investigated by consulting the databases of the Consortium of Functional Glycomics (<http://www.functionalglycomics.org/fg/>) or PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/guide/>). The online Bacterial Carbohydrate Structure Data Base (BCSDB) was consulted to identify the bacterial origin of the glycans (<http://csdb.glycoscience.ru/bacterial/>).

Statistical analysis

Correlation matrixes, heatmaps and hierarchical clustering were performed using “R” (The R Foundation for Statistical Computing, Version 3.0.2)²⁵. Representation of the

186 data was done using the function heatmap.2 from the package *gplots*²⁶. Hierarchical
187 clustering and dendrograms were calculated with the complete method of the hclust
188 function. Statistical analysis and other illustrations were performed using Microsoft
189 Excel (Microsoft Corporation, 2011, Version 14.0.0) and GraphPad PRISM
190 (Graphpad Software, Inc., Version 6.0c). For clique distribution analysis, only groups
191 with common terminal carbohydrate moiety that are represented at least with 12
192 glycans (2% of total) on CFG glycan array version 5.1 were taken into consideration.
193 For statistical analysis, Kruskal-Wallis, Student's *t*-test, Spearman correlation and
194 Two-way ANOVA tests were used. Given the small size of the group (n=2), the CVID
195 PPV^{low} subset on the NCFGv2 array was excluded when group comparisons were
196 performed.

197
198 Further methods are available in the supplemental data, available on the *Blood* Web
199 site.

200 [For original data, please contact the corresponding author.](#)

RESULTS

Broad carbohydrate reactivity defects (CRD) in diverse PAD entities

Sera from PAD patients or healthy donors (HD) were screened on CFG glycan array version 5.1 to analyze the IgG binding reactivity to 610 distinct glycans at 180µg/ml. In accordance with previous reports^{9,13} this assay concentration was determined to be optimal, resulting in reproducible IgG glycan-binding patterns with minimal background. Cohorts included patients with symptomatic hypogammaglobulinemia (HGG n=76), SPAD (n=5) or IgG subclass deficiency (IgGSD; n=8), CVID (n=25), CVID with low PPV vaccination response (CVID PPV^{low}; n=6) or HD (n=43).

Using this platform, we observed broad IgG reactivity to printed glycans in pooled sera from healthy donors (Fig. 1A; Table S2), which is consistent with previously published data^{9,13,21,27-29}. In contrast, marked reduction of overall glycan binding intensities was observed for all investigated PAD patient subsets, with lowest averaged relative fluorescence units (RFU) in CVID, particularly in CVID PPV^{low} patients (Fig. 1A). Next, IgG was purified from CVID, HGG and healthy donor sera by affinity chromatography, and then screened on glycan array. As with whole sera, anti-glycan reactivity of equimolar, purified IgG from immunocompromised patients presented lower RFU values on average compared to healthy controls (Fig. S1).

In an effort to compare the carbohydrate-specific IgG repertoires between PAD patients and healthy subjects, individual sera were screened. To this end glycan array version 2 from the US National Center of Functional Glycomics (NCFGv2) containing 147 glycans was used, which allowed us to include more samples. Identical glycans were similarly recognized on both arrays (Fig. S2). Figure 1B illustrates the computed reactivity matrix (mean RFU values) ordered by a dendrogram clustering algorithm³⁰. Columns represent the antibody reactivity profiles

(reactivity of each specific glycan for the different sera samples), and the rows reflect the immune profiles for each patient subgroup. The dendrogrammed reactivity matrix illustrates the broader spectrum of glycan reactivity in healthy donor sera compared to all screened PAD patient subgroups, yet revealing a similar binding profile as compared to the SPAD cohort. The glycan-binding profiles of IgGSD, HGG and CVID sera displayed a narrower spectrum of glycan reactivity, which was most impaired in the CVID subgroup. CVID and CVID PPV^{low} patients also exhibited the lowest averaged RFU values (Fig. S2). No clear clustering of individual immunoprofiles was observed for total glycans, which was expected as the arrays contain synthetic glycans without known biological relevance (Fig. S2).

Glycan-specific IgG antibodies to bacterial antigens in PAD

PADs are characterized by recurrent, severe or unusual bacterial infections, especially by bacteria protected by a polysaccharide capsule. Thus, we decided to analyze the IgG reactivity profiles to known bacterial carbohydrates. On glycan array CFG version 5.1, we previously identified 121 bacterial carbohydrate antigens consulting the Bacterial Carbohydrate Structure Data Base (BCSDB)⁹, providing a platform for high-throughput analysis for antibody reactivity to carbohydrate epitopes of multiple bacterial species. The printed glycans include capsular and structural oligosaccharides, as well as exopolysaccharides of commensal and pathogenic bacterial species⁹. In comparison to immunoprofiles of healthy subjects, Spearman's rank correlation analysis revealed divergent IgG reactivity profiles to bacterial glycan epitopes in PAD cohorts, with the lowest correlation of 0.34 CVID PPV^{low} patients and 0.57 for CVID, when pooled sera were screened on the CFG array (Fig. 2A). Figure 2B illustrates the collective reactivity matrix (mean RFU values) of individual sera to 65 bacterial glycans on the NCFGv2 array. Hierarchical clustering analysis revealed

a substantial loss of reactivity for the IgGSD, HGG and CVID cohorts. An extensive reduction of anti-bacterial reactivity was also found for recognition of the majority of 121 bacterial glycans on the CFG array version 5.1 in pooled sera from patients in these cohorts (Fig. S3). No clear clustering of individual immunoprofiles was observed for bacterial glycans, which may be due to differential natural exposure to bacteria (Fig. S3).

The glycan array technology combined with the consultation of the BCSDb databank allowed to screen in parallel several distinct epitopes that were identified as glycan constituents of specific bacteria. Figure 2C shows individual profiles of IgG reactivity to glycans on the NCFGv2 array that are found in *E. coli* (glycans: n=15), *H. pylori* (n=15), *N. meningitidis* (n=6), *H. influenzae* (n=3), *S. pneumoniae* (n=2), and *Salmonella* (n=2) species. Heterogeneous immunoprofiles were observed in all cohorts, including healthy individuals. However, reactivity profiles were particularly low in CVID and CVID PPV^{low}, who exhibited a significant loss of IgG antibodies specific for pathogens and commensals, including antigens to which human beings are frequently exposed and immunized. Missing antibodies against *Neisseria meningitidis* and *Salmonella* in the different groups may reflect lack of exposure to these pathogens.

Recognition of TACA and Siglec ligands in PAD

One life-threatening complication of PAD, in particular of CVID, is the occurrence of malignancies^{1,31-34}. Since altered surface glycosylation is a hallmark of cancer and influences different aspects of tumor progression and anti-tumor immunity^{7,35,36}, we set out to explore IgG reactivity to tumor-associated carbohydrate antigens. Figure 3A demonstrates the IgG reactivity profiles to twenty-two glycan epitopes on CFG glycan array version 5.1 that constitute well-established TACAs. In

277 accordance with our previous data⁹, healthy donor sera contained natural antibodies
278 against TACAs. High reactivity was found against Lewis antigen-related TACAs,
279 gangliosides, Thomsen-Friedenreich (TF) and globo-series-associated antigens.
280 Loss of anti-TACA IgG reactivity was found in all examined PAD patient subgroups.
281 The reduced binding activity by IgG was most dominant in CVID PPV^{low} donors,
282 followed by CVID patients, as computed based on RFU profiles and represented as
283 circular dendrogram (Fig. 3B). To further explore these findings we analyzed anti-
284 TACA signatures of individual sera screened on the NCFGv2 array. Most sera from
285 healthy individuals exhibited high anti-TACA reactivity to Forssman antigen,
286 Globoside, Globo H and SSEA-4 hexaose, but heterogenous binding to other TACAs
287 (Fig. 3C). The substantial loss of anti-TACA reactivity in CVID was confirmed in
288 individual sera. In contrast, antibody binding to the Forssman antigen was found in all
289 cohorts and all screened individual sera (Fig. 3A and C), indicating that this glycan-
290 epitope is highly immunogenic with widely maintained specific antibody production in
291 the PAD patients. Indeed, in the CVID PPV^{low} and some CVID patients, Forssman
292 antigen was the only investigated TACA for which higher intensity signals were
293 detected. Reactivity to GM1 was particularly low in CVID patients. This is remarkable
294 as GM1 expression is present and positively correlates with rituximab treatment
295 response in non-Hodgkin's lymphoma³⁷, which is among the most frequent
296 malignancies occurring in these patients¹⁰.

297 Overexpression of sialylated TACAs frequently occurs in tumors and
298 hypersialylation has been linked to tumor progression and immune escape by the
299 engagement of inhibitory Siglec receptors on leukocytes^{7,36,38-40}. In PAD cohorts the
300 antibody recognition of certain sialoglycans with known Siglec binding capacity was
301 similar or reduced on the glycan array compared to healthy donor responses (Fig.
302 S4). However, for two ligands of CD22/Siglec-2, expressed on B cells, binding was

high (# 377) or even increased (# 268). Little is known about the tissue expression of these two ligands and it remains to be shown if such antibodies at higher levels might interfere with the CD22 receptor / ligand axis to unleash B cell responses under certain circumstances.

Failure to raise Gal α - and GalNAc-directed IgG antibodies in PAD

The composition of glycan antigens, in particular the terminal carbohydrate-moiety, has been linked to the immunogenicity of a given glycan in healthy individuals⁹. This raised the question about the effect of the relationship between immune system failure (ISF) in PAD subgroups and the structure-related immunogenicity on the architecture of the glycan-specific IgG repertoire. A binary deviation matrix was computed considering statistical deviation ($P < 0.05$) of IgG binding (RFU values) for each PAD cohort and each glycan compared to healthy donor data and reordered by a dendrogram clustering algorithm (Fig. 4A). The rows in this matrix indicate the binary antibody reactivity profiles, and the columns represent the deviation immune profiles for each PAD subgroup. By hierarchical clustering analysis four major subgroups were identified: predominant were clique 4 representing glycans with deviation in all PAD cohorts (n=293; 48%) and clique 2 containing glycans without deviation in all PAD cohorts (n=245; 40%); of lower magnitude were clique 1 (n=5; 1%) and clique 3 (n=67; 11%) encompassing glycans with heterogeneous IgG binding activities between subgroups (Table S3).

Given the association between immunogenicity and terminal carbohydrate moiety of glycans^{9,41}, the clique distribution of glycans was investigated based on their structure. The most dominant deviation was found for Gal α - and GalNAc-terminated glycans (Fig. 4B). Forty-seven (85.5%) versus only one (1.8%) Gal α -structure(s), and 37 (68.5%) versus 12 (22.2%) GalNAc-terminated glycans were

represented in clique 4 or clique 2, respectively. In accordance, the IgG antibody-binding levels (RFU values) of Gal α - and GalNAc-terminated glycans were lower in all cohorts (Fig. 4C, D). In depth analysis computing a deviation matrix based on *P* values and hierarchical clustering analysis revealed large glycan clusters that were concomitantly either non-aberrant (clique B) or aberrant (clique E) for all disease entities compared to healthy individuals (Fig. 4E), whereby Gal α - (70.9%) and GalNAc- (62.9%) terminated glycans were most prevalent in clique E (Fig. 4F), indicating failed antibody responses to these specific structures.

As a consequence of the inactivation of the *GGTA1* gene encoding for α 1,3galactosyltransferase (GalT), humans, apes and Old World monkeys do not express the Galili epitope Gal α 1-3Gal β 1-4GlcNAc β ³⁹. Natural antibodies to this xenoantigen in most humans, eventually generated in response to the microbiota of the host, are considered a key factor in the rejection of xenografts⁴³, and necessitated the development of GalT knockout (GalTKO) animals^{44,45}. Using a suspension array, we examined individual sera from patients (HGG, n=37; CVID, n=15; IgGSD, n=8) and healthy donors (n=18) for IgG reactivity to the Galili epitope and other Gal α - and GalNAc-terminated structures including, α LN (Gal α 1-4GlcNAc β), A α 3GN (Gal α 1-3GlcNAc β), or A and B blood group antigens (Fig. 5A and S5). Low isohemagglutinins are frequently observed in CVID patients¹¹. Consistent with this observation, we found reduced IgG reactivity to blood group A and B antigens also in the HGG, SPAD and IgGSD cohorts (Fig. S6). The IgG reactivity to Gal α - and GalNAc-terminated epitopes was consistently lower in most PAD patients, whereby the reduction was most significant for the Galili epitope, as evidenced by statistical analysis (Fig. 5A and S5).

Given the significant loss of anti-Galili reactivity in PAD patients, functional implications were tested in a xenoreactivity assay, analyzing antibody-dependent cell cytotoxicity (ADCC) of primary human natural killer (NK) cells directed against the porcine kidney cell line PK15. On this cell line the Galili epitope is highly expressed on the surface and lost following enzymatic digestion by α -galactosidase as assessed by flow cytometry (Fig. 5B). Sera from healthy donors induced substantial NK cell-mediated ADCC activity against the Galili-positive PK15 cells, which was abolished following α -galactosidase treatment of the porcine target cells (Fig. 5C), illustrating the dependence of the xenogeneic activity on Gal- α epitopes, including Galili. In contrast, CVID sera failed to promote NK cell-mediated ADCC against the porcine Galili-positive target cells at equimolar IgG concentrations (1mg/mL), indicating a repertoire defect for the recognition of xenogeneic antigens. Moreover, the loss of the xenogeneic potential of CVID sera was demonstrated in a modified version of an established model for antibody-mediated skin damage^{46,47}, involving cryosections of porcine skin incubated with patients' sera and leukocytes from healthy donors and the assessment of dermal-epidermal separation. Porcine skin damage was significantly lower in the presence of CVID sera compared to human control sera at equimolar IgG concentrations (Fig. 5D). Together, these data provide evidence for humoral immune system failure beyond quantitative antibody deficiency, which involves repertoire defects in PAD.

DISCUSSION

The emerging picture is that the human IgG anti-carbohydrate repertoires in health⁹, and in primary antibody deficiencies, exhibit a modular organization, yet with a different architecture in PAD. Translated to Cohen's concept of immune computation⁴⁸, our data imply that primary antibody deficiency is not solely characterized by aberrant antibody production, but features disturbed immune-system response states (the output) due to an altered algorithm to compute immunogenic states (the input) that is dictated by impaired cellular and molecular networks of innate and adaptive immunity^{49,50}.

Despite the heterogeneous genetic basis across PAD entities¹, deviation mapping revealed significant repertoire similarities pointing toward a common disease-modifying algorithm that drives humoral immune system failure. Dominant was the reduced or lost specificity for Gal α - or GalNAc-terminated glycan epitopes in PAD patients, for which healthy individuals express high levels of naturally occurring antibodies, eventually in response to immune stimulation by carbohydrate antigens of their microbiota^{9,41}. The loss of Gal α - or GalNAc-specific antibodies might thus reflect the lost capacity to induce these antibodies in response to the host microbiota, or reflect the microbial dysbiosis in PAD⁴, or both. Interestingly, bacteria and tumor cells use certain glycosylation patterns to escape immunity, such as sialoglycans that engage inhibitory Siglec receptors on leukocyte subsets of myeloid and lymphoid lineages^{6,35,39,40,51}. It remains to be shown if naturally-occurring antibodies to TACA are also induced by the microbiota and if the observed loss has consequences for PAD patients. Notably, the loss of IgG reactivity to tumor antigens was most evident in the CVID cohort, a disorder in which the incidence of malignancy is increased¹⁰, supporting the concept of aberrant immune surveillance in PID⁵². For instance, we

398 observed low antibody reactivity to TACAs known to be expressed on CVID-
399 associated malignancies, such as GM1 in non-Hodgkin's lymphoma³⁷, or Thomson-
400 Friedenreich antigen in gastric cancer^{53,54}. However, tumor glycosylation patterns and
401 their immunoreactivity remain to be fully explored for CVID-associated malignancies.

402 Glycoimmunology remains a relatively underdeveloped field and the need for
403 interdisciplinary collaborations and the enhanced training of glycoscientists in
404 biomedical areas has been identified⁵⁵. In line with this, there is also a demand for
405 tools, such as glycan arrays, the development of which is challenging given the
406 availability of differently synthesized or purified glycans. However, this initial study
407 highlights the potential and the need to expand glycan array studies in PAD using
408 biological samples from larger patient cohorts. Clusters of glycans were identified
409 with different reactivity profiles between PAD subgroups (cliques 3, A, C, D), which
410 may be candidates for diagnostic use. In future studies, it will also be advisable to
411 compare glycan-specific antibody profiles with clinical and laboratory parameters
412 (e.g. switched memory B cell numbers, blood groups), and in specifically classified
413 patient subsets⁵⁶.

414 The broad and qualitative assessment of the immune system failure (ISF) in
415 individual patients may be informative regarding the severity of the disease,
416 eventually leading to more personalized treatment decisions. Glycan array
417 technology may thus provide a useful option for the individual assessment of ISF and
418 facilitate clinical practice, especially given the heterogeneity of PAD entities with a
419 growing list of identified pathogenetic defects². However, our broad analysis
420 demonstrates the extent of the skewed IgG repertoire with impaired reactivity to
421 biologically relevant glycan epitopes, including those linked to PAD-associated
422 clinical manifestations, such as infection, malignancy and autoimmunity^{1,10}. Our

systems immunology approach highlights the power of high-throughput assessment of humoral immune system failure by microarray technology, which may have potential ramifications for the diagnosis, classification and therapy of PAD patients.

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AUTHORSHIP

Contributions: S.V.G. and P.J. designed the study. K.F.B., P.J. and S.V.G. wrote the manuscript. K.F.B. and S.V.G. analyzed the data. Glycan array experiments at the CFG were conducted under supervision of D.F.S. and R.D.C. Database searches

and computational analysis of the data set was performed by K.F.B. Patient sample collection, classification and preparation were done by B. G., C.J. and P.J. Experimental work was done by K.F.B., E.D.G., M.A., A.D. under supervision by D.S., H.U.S., R.R. and S.V.G. Glycan synthesis was supervised by N.B. All authors had full access to the data, helped draft the report or critically revised the draft, contributed to data interpretation, reviewed and approved the final version of the report.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Antibody repertoire profiling on CFG and NCFGv2 glycan arrays reveals broad carbohydrate recognition defects in symptomatic primary antibody deficiencies (PADs).

(A) Glycan-specific binding of serum IgG screened at 180 μ g/ml on CFG glycan array version 5.1 (610 glycans) depicted as relative fluorescence units (RFU). Screened sera were from cohorts with symptomatic hypogammaglobulinemia (HGG; n=76), specific antibody deficiency (SPAD; n=5), common variable immunodeficiency (CVID; n=25), CVID with low PPV vaccination response (CVID PPV^{low}; n=6), IgG subclass deficiency (IgGSD; n=8), or healthy donors (HD; n=43). Significant values are reported, Kruskal–Wallis test. **(B)** Glycan-binding reactivity matrices for individual sera (HD: n=12; SPAD: n=5; IgGSD: n=8; HGG: n=11; CVID: n=12) screened on the NCFGv2 array and computed by the dendrogram clustering algorithm as outlined in Materials and Methods. The color key and distribution histogram are depicted. Columns represent the antibody reactivity profiles (reactivity of each specific glycan for the different sera samples), and the rows reflect the immune profiles for each patient subgroup, based on the mean relative fluorescent units (RFU) values.

Figure 2. Recognition of bacterial carbohydrate epitopes in PADs.

(A) Spearman's rank correlation matrix for IgG recognition of pooled sera to all 121 bacterial carbohydrate structures identified by BCSDB analysis on the CFG array.

(B,C) Recognition of bacterial glycans by individual sera screened on the NCFGv2 array. **(B)** Dendrogrammed reactivity matrix based on the mean RFU values for all 65 glycans identified by BCSDB analysis. The color keys and distribution histograms are depicted. **(C)** Heatmap presentation of glycan-specific IgG binding to selected

epitopes from *S. pneumoniae* (n=2), *H. influenzae* (n=3), *N. meningitidis* (n=6), *E. coli* (n=15), *H. pylori* (n=15), and *Salmonella* (n=2) species.

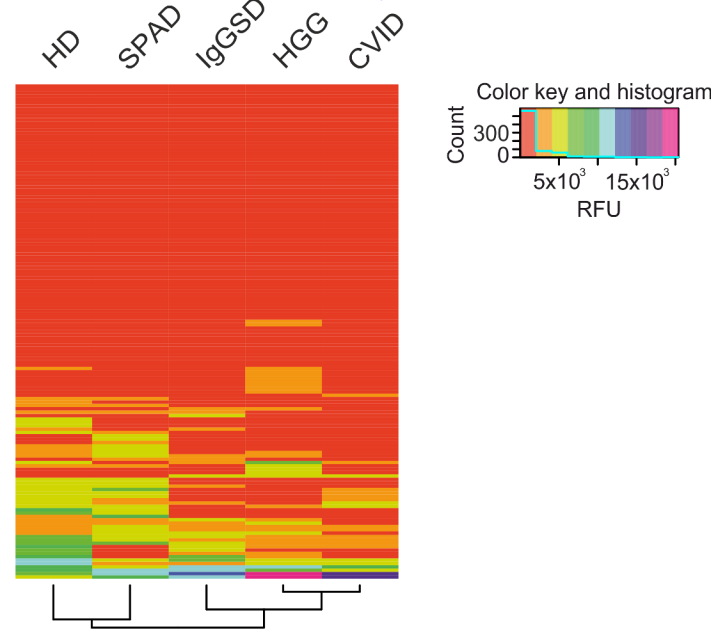
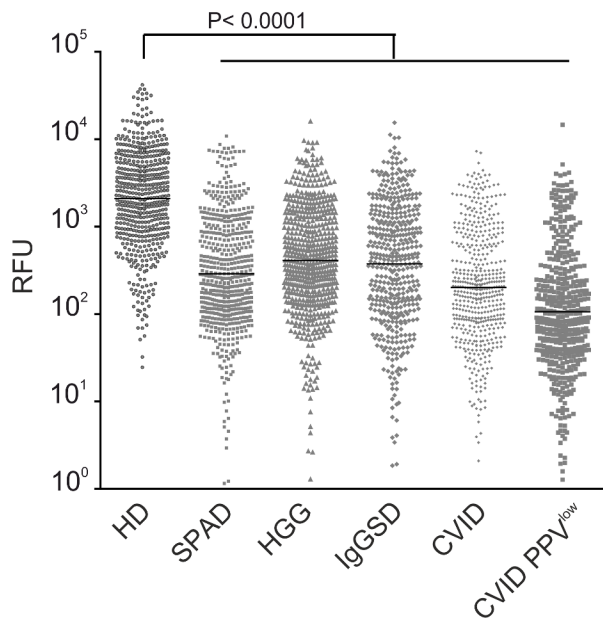
Figure 3. Recognition of tumor-associated carbohydrates (TACAs) by glycan-specific IgG in PAD. (A, B) Recognition of TACAs by pooled serum IgG as screened on the CFG array and depicted as dendrogrammed glycan reactivity matrix based on ABR **(A)**, or as radial dendrogram based on RFU values **(B)**. **(C)** Heatmap presentation based on RFU values illustrating reactivity of individual serum IgG to TACAs as revealed by screening on the NCFGv2 array. The color keys and distribution histograms are depicted.

Figure 4. Deviant recognition of distinct terminal carbohydrate moieties and specific loss of Gal α reactivity in symptomatic PAD. (A) Binary deviation map (P < 0.05) of IgG immunoprofiles compared to healthy controls based on two-way ANOVA and hierarchical clustering analysis with identification of cliques 1-4. The rows in this matrix indicate the binary antibody reactivity profiles, and the columns represent the deviation immune profiles for each PAD subgroup. **(B)** Terminal carbohydrate moieties of epitopes in reactivity cliques 2 (non-significant deviation) and 4 (significant deviation). Numeric occurrence is indicated in parenthesis. **(C, D)** Recognition of Gal α - **(C)** or GalNAc- **(D)** epitopes within clique 4 (significant deviation). **(E)** Deviation map based on degree of significance with identification of cliques A-E. **(F)** Bubble chart displaying terminal glycan structure distribution across cliques A-E. The size key and percentage numbers indicate frequencies.

Figure 5. Diminished recognition of Gal α -terminated glycan epitopes and reduced Gal α -dependent xenogeneic anti-porcine reactivity of CVID and symptomatic IgGSD sera. (A) IgG antibody reactivity to Galili, α LN and A α 3GN

glycans as assessed by suspension array (multiplex immunoassay). Individual sera from healthy donors (n=18), HGG patients (n=37), CVID (n=15) and IgGSD (n=8) patients were analyzed. Box-and-whisker diagrams were created by the Tukey method. The middle line is plotted at the median and the box represents the interquartile range (difference between the 25th and 75th percentiles). Significant values are reported, Kruskal–Wallis test. **(B)** Reduced surface staining of anti-Galili mAb reactivity following α -galactosidase treatment of porcine PK15 cells, as assessed by flow cytometry. Representative histogram (left panel) and summary (right panel). **(C)** Antibody dependent cellular cytotoxicity (ADCC) activity of primary human NK cells against PK15 cells at E/T ratio of 10:1, in absence or presence of α -galactosidase. Significant values are reported (Student's *t*-test). **(D)** Histopathologic analysis of pig skin damage in cryosections incubated with healthy and patient sera in presence of healthy human leukocytes. Representative examples for each analyzed disease and control (PBS) are shown (left panel). The dashed lines indicate the dermal-epidermal junction. Pictures were taken with 40X magnification, scale bars 75 μ m. The damage induced was calculated as the dermal-epidermal separation normalized to the IgG concentration in the sera (right panel) (at least n=7). Results are representative of at least three **(C)** or seven **(D)** experiments. Significant values are reported (Kruskal–Wallis test).

A



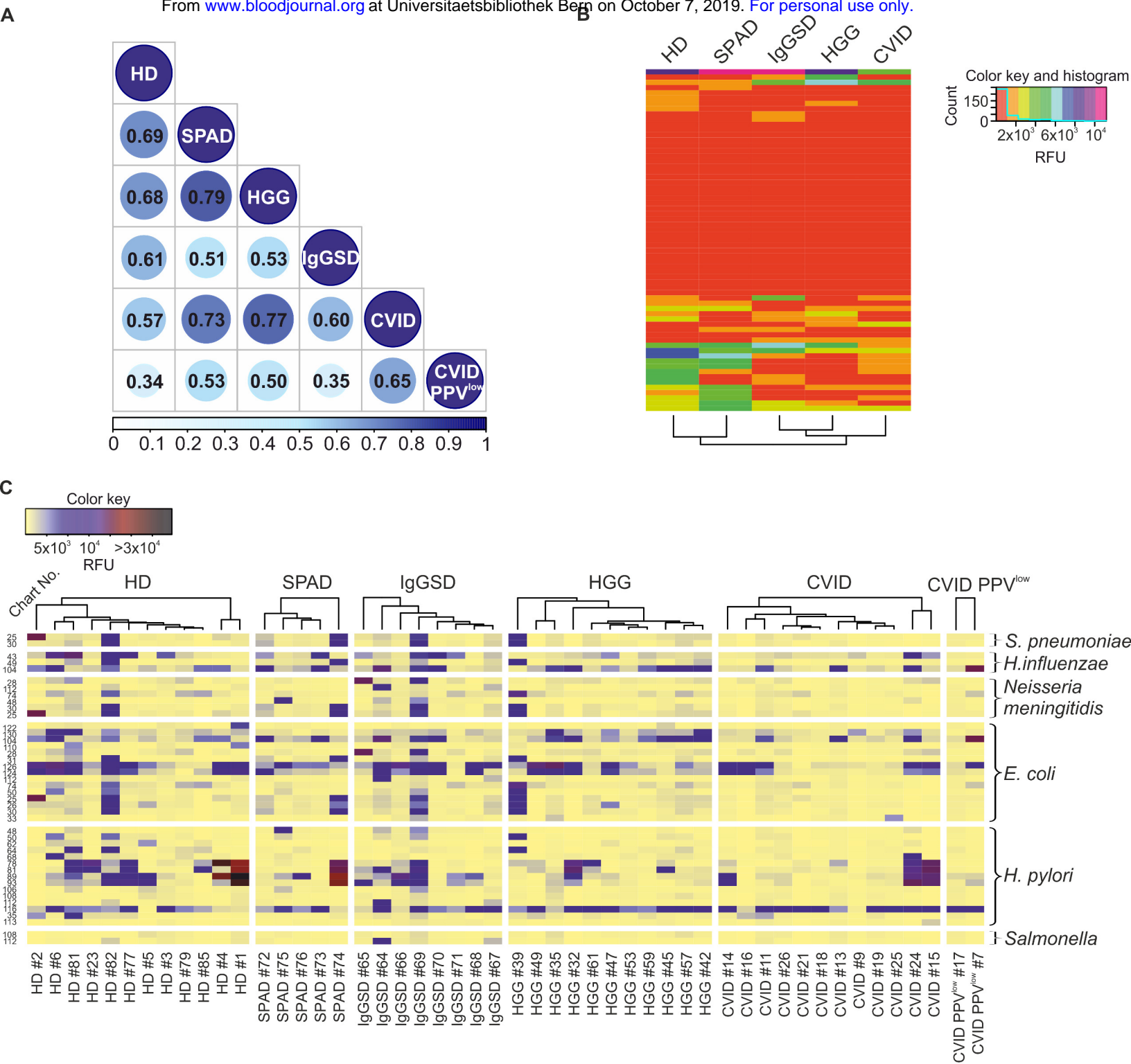


Figure 2

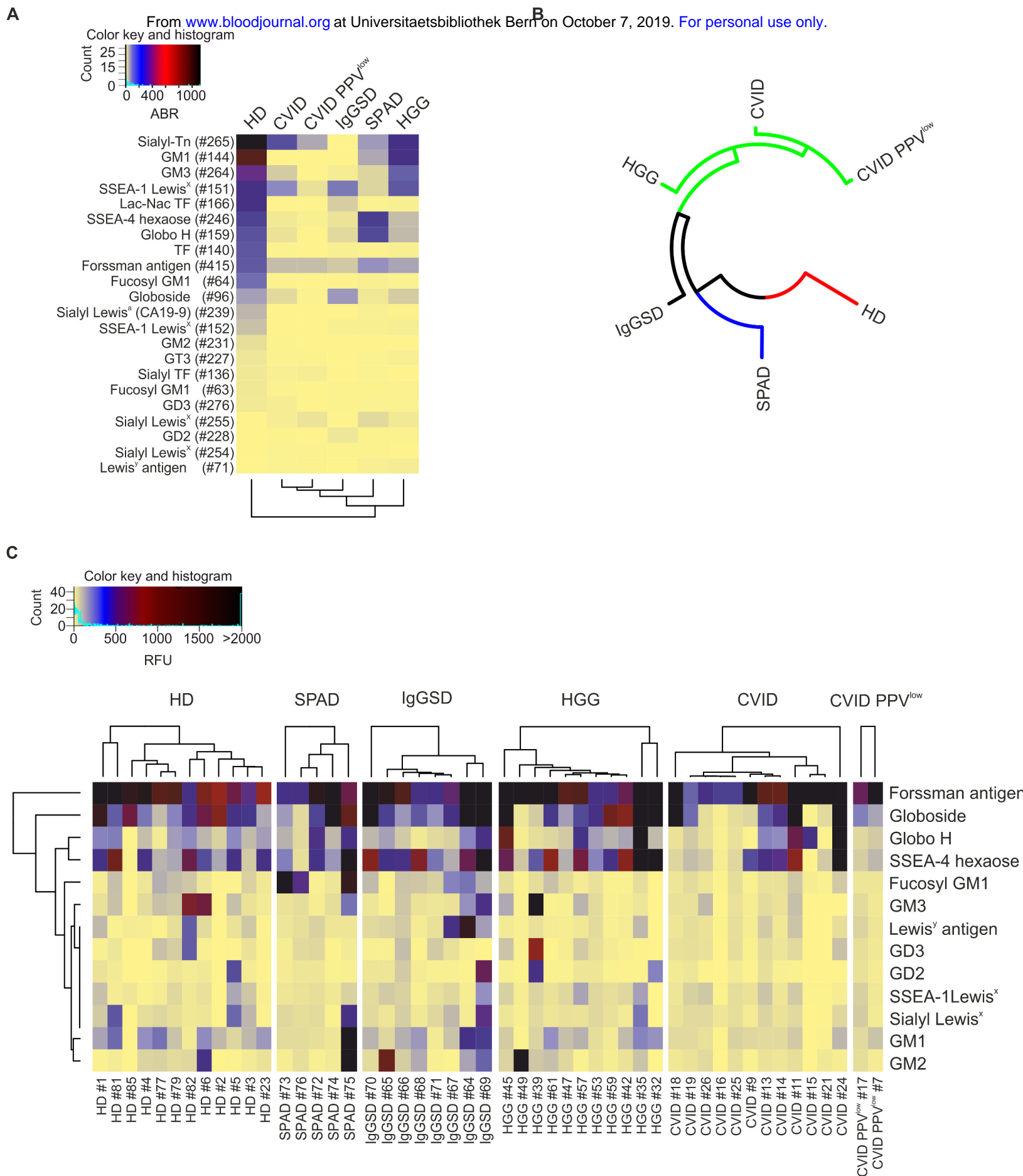


Figure 3

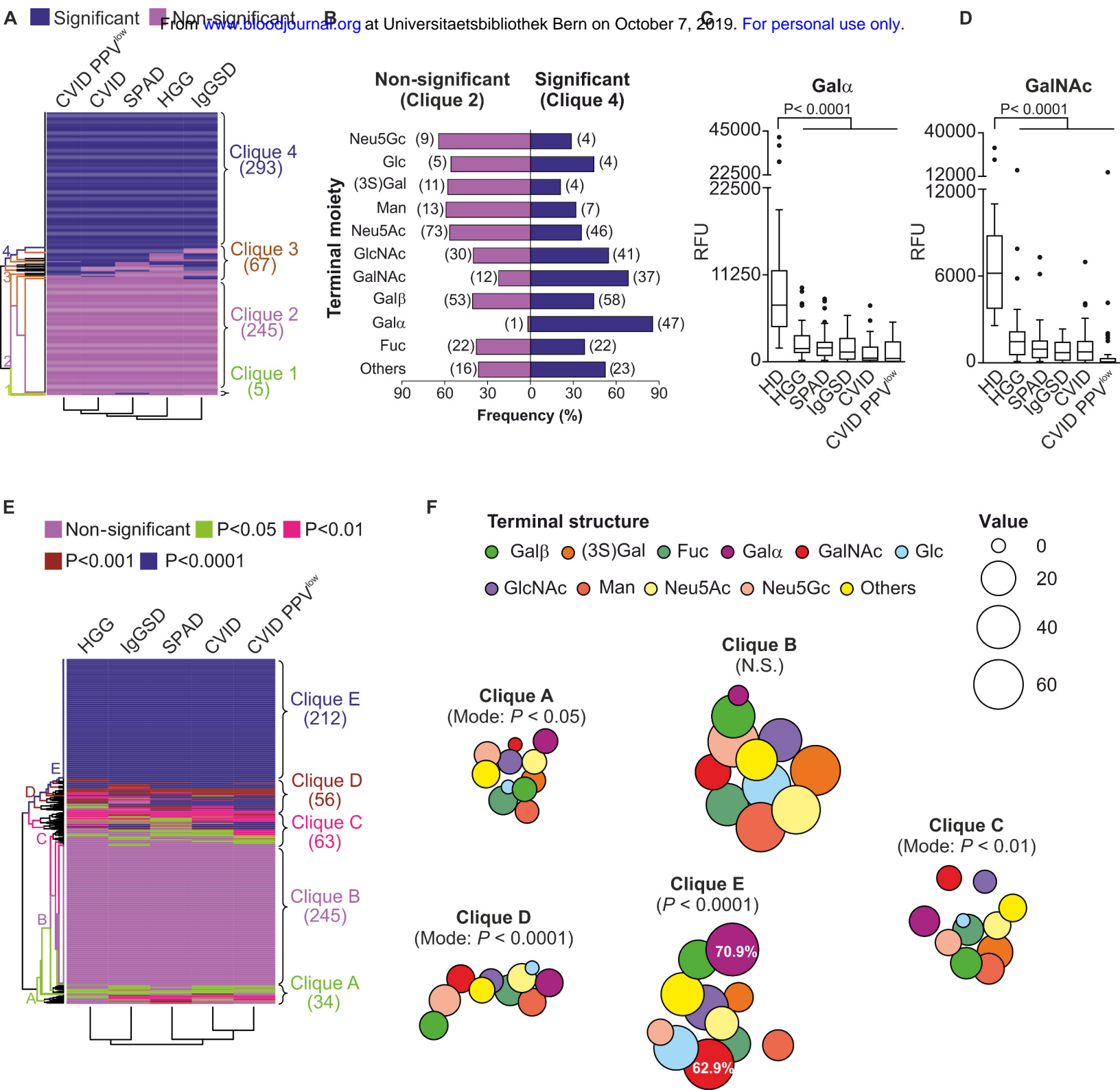


Figure 4

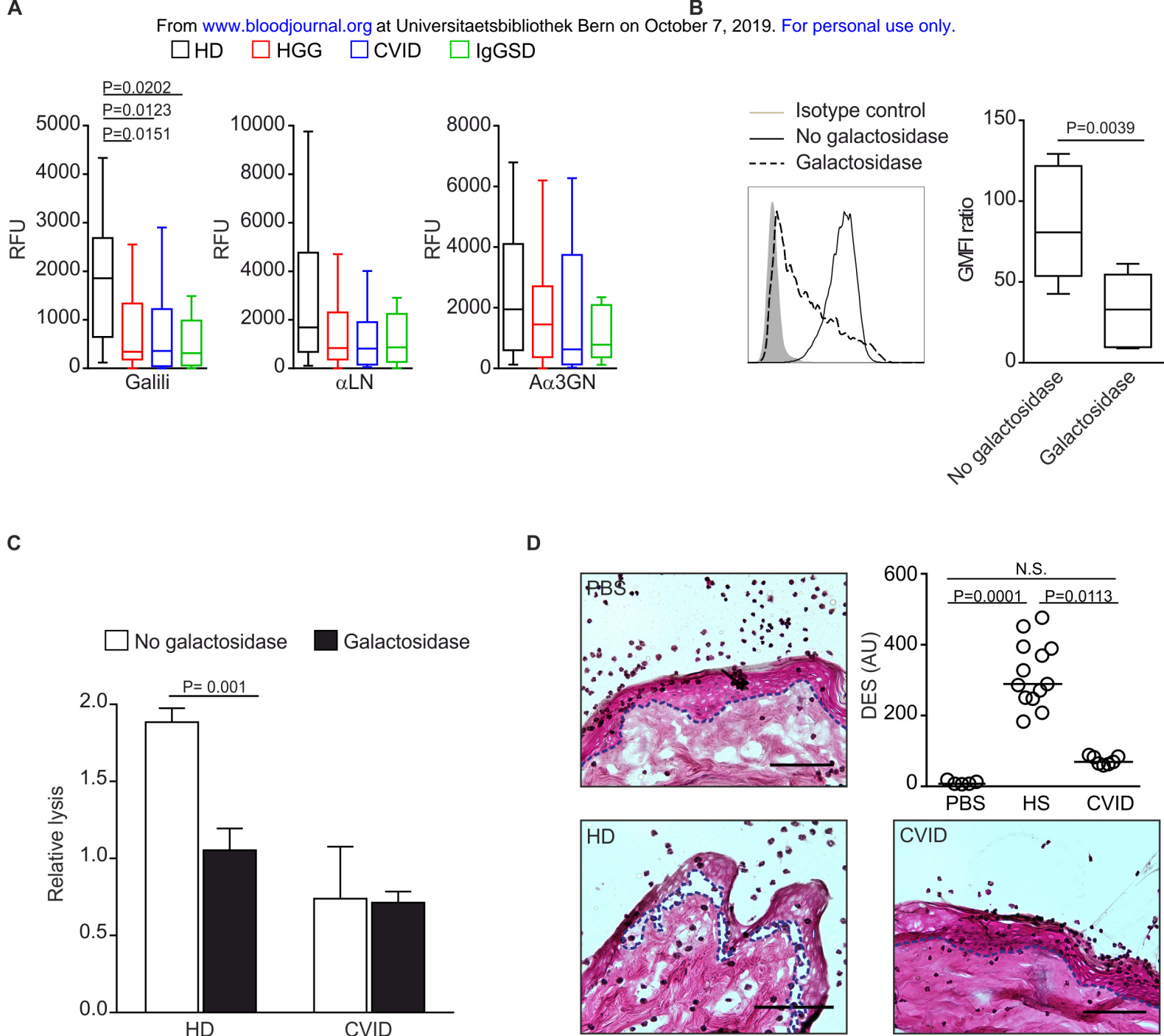


Figure 5



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The architecture of the IgG anti-carbohydrate repertoire in primary antibody deficiencies (PADs)

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